Product Description SALSA® MLPA® Probemix P055-D1 PAH

To be used with the MLPA General Protocol.

Version D1. For complete product history see page 7.

Catalogue numbers:
- **P055-025R:** SALSA MLPA probemix P055 PAH, 25 reactions.
- **P055-050R:** SALSA MLPA probemix P055 PAH, 50 reactions.
- **P055-100R:** SALSA MLPA probemix P055 PAH, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman capillary sequencers, respectively (see www.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

Intended use: SALSA® MLPA® Probemix P055 is an in vitro diagnostic (IVD)[1] or research use only (RUO) assay for the identification of the cause of phenylalanine hydroxylase deficiency in patients diagnosed for phenylketonuria (PKU). The probemix detects deletions/duplications of one or more exons of the PAH gene. When a deletion or duplication has been identified in patient DNA, this product can also be used for carrier screening of family members.

This assay is optimised for use with peripheral blood derived genomic DNA and DNA from cells collected with buccal swabs. Deletions or duplications obtained with the P055 PAH probemix must be verified by another technique. In particular, copy number changes detected by only a single probe always require validation by another method. This SALSA® MLPA® probemix is not intended to be used for initial diagnosis of PKU or as a standalone assay for clinical decisions. Most defects in the PAH gene are point mutations, the majority of which will not be detected by MLPA. It is therefore recommended to use this SALSA® MLPA® probemix in combination with sequence analysis. The results of this test are intended to be interpreted by a clinical molecular geneticist or equivalent.

[1]Please note that this probemix is for In Vitro Diagnostic use (IVD) in the countries specified at the end of this product description. In all other countries, the product is for Research Use Only (RUO).

Clinical background: Phenylketonuria (PKU), as well as the less severe forms of the condition (sometimes called variant PKU and non-PKU hyperphenylalaninemia), is an inborn error of metabolism characterised by hyperphenylalaninemia resulting from a deficiency of phenylalanine hydroxylase (98% of cases) or impaired synthesis or recycling of tetrahydrobiopterin (2% of cases) (Scriver and Kaufman 2001). Untreated PKU can lead to microcephaly, epilepsy, severe mental retardation and behavioural problems. More information on PKU is available on http://www.ncbi.nlm.nih.gov/books/NBK1504/.

The majority of PKU cases are due to defects in the PAH gene. Among these defects are deletions and duplications of complete exons, which are usually missed by standard sequence analysis. The MLPA technique can detect most of these deletions and duplications and therefore complements sequence analysis of the PAH gene. The expected number of PAH chromosomal rearrangements that can be detected with this MLPA probemix is between 1 and 5% of all PKU cases in most populations (see below for publications on probemix P055 PAH).

Gene structure: The *PAH* gene spans 90 kb on chromosome 12q23.2, about 103 Mb from the p-telomere, and contains 13 exons. No LRG sequence (www.lrg-sequence.org/) for PAH is available at this moment. The GenBank chromosomal DNA sequence is NG_008690.1.

Transcript variants: Only one PAH transcript variant has been defined: NM_000277.1 (2680 nt, coding sequence 473-1831, www.ncbi.nlm.nih.gov/gene/5053).

Exon numbering: The exon numbering used in this P055-D1 PAH product description and in the P055-D1 PAH Coffalyser.Net analysis sheet is identical to the exon numbering in the NCBI NG_008690.1 reference sequence. The exon numbering and NM sequence used is from 06/2018, but can be changed (e.g. by NCBI) after the release of the product description.

Probemix content: This SALSA® MLPA® probemix P055 PAH contains 38 MLPA probes with amplification products between 128 and 427 nt including 22 probes for the PAH gene region, one probe upstream and downstream of PAH (Table 2), and 14 reference probes that detect sequences outside this region. The identity of the genes detected by the reference probes is available online (www.mlpa.com).

Several partially conserved and possibly regulatory elements are located just upstream of PAH exon 1. These DNaseI hypersensitive sites have been described by Bristeau et al. (2001). Two probes are located in these upstream sequences (418 nt HSS3 and 154 nt HSS2).

This Probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity Fragments (Q-fragments), two DNA Denaturation Fragments (D-fragments), one benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (Only visible with <100 ng sample DNA)
88-96	D-fragments (Low signal of 88 or 96 fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.com).

MLPA technique validation: Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation <0.10 for all probes over the experiment.

Required specimens: Extracted DNA from human peripheral blood or buccal swabs, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples: All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from unrelated individuals who are from families without a history of phenylketonuria. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

Positive control DNA samples: MRC-Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Biobank (https://catalog.coriell.org) and DSMZ (https://www.dsmz.de/home.html) have a diverse collection of biological resources which may be used as a

positive control DNA sample in your MLPA experiments. The quality of cell lines can change, therefore samples should be validated before use.

Performance characteristics: The expected number of PAH chromosomal rearrangements that can be detected with this MLPA probemix is between 1 and 5% of all PKU cases in most populations (see below for publications on probemix P055 PAH). The analytical sensitivity and specificity for the detection of deletions/duplications in the PAH gene is very high and can be considered >95%.

Analytical performance can be compromised by: SNPs or other polymorphisms (e.g. indels) in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis: Coffalyser.Net software must be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mlpa.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results: The expected results for PAH region specific MLPA probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 0 (homozygous deletion), 3 (heterozygous duplication) and occasionally 4 (homozygous duplication or heterozygous triplication).

The standard deviation of all probes in the reference samples should be <0.10 and the dosage quotient (DQ) of the reference probes in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the DQ of the probes can be used to interpret MLPA results for autosomal or pseudo-autosomal chromosomes:

Copy Number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/ Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the PAH gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might in some cases not result in inactivation of that gene copy.

- Copy number changes detected by reference probes are unlikely to have any relation to the condition tested for.

Notes PAH results:
- PKU is caused by defects in the PAH gene and is an autosomal recessive disease. Inactivation of both copies of the PAH gene is expected to result in PKU. Inactivation of only a single copy of the PAH gene is typically seen in carriers.
- Deletion of one or more exons usually results in inactivation of that gene copy. Single exon deletions however have a considerable chance of being a false positive result. A 3.7 kb deletion in the 5'-flanking region of the PAH gene, including the DNaseI hypersensitive sites (HSS2 and HSS3 region, described by Bristeau et al. (2001)) has been found in a hyperphenylalaninemic patient (Chen et al. 2002).
- Duplication of an internal part of a gene usually results in a defective copy of that gene, as the duplicated sequence is typically located directly adjacent to the original sequence, resulting in a defective transcript. Duplication of the complete PAH gene is not expected to result in disease.
- Deletion or duplication of the flanking probes for ASCL1 and IGF1 are not expected to be the cause of PKU. These probes have only been included to delineate the extent of large deletions and duplications.

Limitations of the procedure:
- In most populations, the major cause of genetic defects in the *PAH* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA probemix P055 PAH.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

Confirmation of results: Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MLPA result was obtained.

Copy number changes detected by one or more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

PAH mutation database: http://www.pahdb.mcgill.ca/. We strongly encourage users to deposit positive results in the PAH of Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNPs and unusual results (e.g., a duplication of *PAH* exons 6 and 8 but not exon 7) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P055-D1 PAH

Length (nt)	SALSA MLPA probe	Chromosomal position(a) reference	PAH
64-105	Control fragments – see table in probemix content section for more information		
128	Reference probe 00797-L00093	5q31	
136	Reference probe 07292-L06929	6q16	
142	**PAH** probe 02326-L01823		**Exon 7**
149 « ¬	**ASCL1** probe 02327-L01835		**Telomeric**
154	**HSS2** probe 12251-L14053		**Upstream**
161	**PAH** probe 02328-L11413		**Exon 8**
168	**PAH** probe 16487-L23233		**Exon 1**
174	Reference probe 01823-L23229	16p13	
180	**PAH** probe 16488-L23230		**Exon 2**
187	**PAH** probe 02331-L23231		**Exon 9**
194	Reference probe 05703-L06959	3q21	
201	**PAH** probe 16489-L18945		**Exon 3**
211	**PAH** probe 02333-L01826		**Exon 10**
220	Reference probe 01782-L01346	13q14	
227	**PAH** probe 17737-L21083		**Exon 6**
235	**PAH** probe 02334-L23232		**Exon 4**
242	**PAH** probe 02335-L14055		**Exon 11**
247	Reference probe 07695-L07419	21q22	
256	**PAH** probe 02336-L01821		**Exon 5**
265	**PAH** probe 02337-L02469		**Exon 12**
274	Reference probe 15473-L17313	1p36	
283	**PAH** probe 16491-L18947		**Exon 6**
292	**PAH** probe 02339-L01829		**Exon 13**
300	Reference probe 01575-L01147	22q12	
310 ¬	**IGF1** probe 02340-L01834		**Centromeric**
319	Reference probe 06440-L05966	3P12	
337	**PAH** probe 12254-L14056		**Exon 1**
346	**PAH** probe 16492-L18948		**Exon 5**
352	**PAH** probe 12256-L14058		**Exon 7**
359 Ж	Reference probe 13731-SP0136-L15212	15q11	
365 Ж	**PAH** probe 16493-SP0373-L18949		**Exon 3**
373	**PAH** probe 16494-L18950		**Exon 2**
382	Reference probe 13974-L15543	7q34	
391	Reference probe 12522-L13572	18q21	
400	**PAH** probe 12260-L14061		**Exon 4**
409	Reference probe 10063-L10487	8q22	
418	**HSS3** probe 12261-L13203		**Upstream**
427	Reference probe 05915-L17921	14q11	

(a) The exon numbering used in this P055-D1 PAH product description and in the P055-D1 PAH Coffalyser.Net analysis sheet is identical to the exon numbering in the NCBI NG_008690.1 reference sequence. The exon numbering and NM sequence used is from 06/2018, but can be changed (e.g. by NCBI) after the release of the product description.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of flanking and reference probes are unlikely to be related to the condition tested.

Table 2. PAH probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / Exon[a]	Ligation site[b] NM_000277.1	Partial sequence[c] (24 nt adjacent to ligation site)	Distance to next probe
149 « ¬	02327-L01835	ASCL1 gene		ACCTGCATCTTT-AGTGCTTTCTTG	38.9 kb
418	12261-L13203	HSS3 region	3352 nt before exon 1	CAATGGTTGGGT-AATCTTCAACTT	2.0 kb
154	12251-L14053	HSS2 region	1393 nt before exon 1	GTGGTAGAACCA-AGAGTTAAACCA	1.0 kb
		PAH start codon	*473-475 (exon 1)*		
337	12254-L14056	PAH exon 1	68-69	GGCTTAGTCCAA-TTGCAGAGAACT	0.4 kb
168	16487-L23233	PAH exon 1	430-431	CTGCCTGTACCT-GAGGCCCTAAAA	4.1 kb
373	16494-L18950	PAH exon 2	191 nt before exon 2	GTAGCATCATTG-ATCATTTAATTG	0.4 kb
180	16488-L23230	PAH exon 2	73 nt after exon 2	AGTTAGATGCAA-TGAAAAGAACAC	17.6 kb
365 Ж	16493-SP0373-L18949	PAH exon 3	235 and 201 nt before exon 3	ATTTTCATGTGA-34 nt spanning oligo-CCTGCCACTTAG	0.4 kb
201	16489-L18945	PAH exon 3	33 nt after exon 3	CAACATAAGTAA-CTCCACACTGTC	17.2 kb
235	02334-L23232	PAH exon 4	842-843	GGTTCCCAAGAA-CCATTCAAGAGC	0.1 kb
400	12260-L14061	PAH exon 4	3 nt after exon 4	GACCACCCTGTG-AGTCCATGGCCC	10.7 kb
346	16492-L18948	PAH exon 5	104 nt before exon 5	CCAAGGGAAGGA-GACATGCACTGT	0.2 kb
256	02336-L01821	PAH exon 5	971-972	ACATTGCCTACA-ACTACCGCCAGT	11.2 kb
283	16491-L18947	PAH exon 6	106 nt before exon 6 reverse	TGAGCTGCCATC-ACTTGCTACAGT	0.2 kb
227	17737-L21083	PAH exon 6	1023-1022 reverse	CCCATGTTTTCT- TTTCTTCCTCCA	2.4 kb
142	02326-L01823	PAH exon 7	1235-1236	CTCGGGATTTCT-TGGGTGGCCTGG	0.1 kb
352	12256-L14058	PAH exon 7	37 nt after exon 7	TTGCCAGGCACA-ATGAGCGCCATC	1.1 kb
161	02328-L11413	PAH exon 8	1344-1345	GTTGGGACATGT-GCCCTTGTTTTC	4.8 kb
187	02331-L23231	PAH exon 9	1425-1424 reverse	CGAGCTTTTCAA-TGTATTCATCAG	2.5 kb
211	02333-L01826	PAH exon 10	1500-1501	CATAAAGGCATA-TGGTGCTGGGCT	0.6 kb
242	02335-L14055	PAH exon 11	1588-1589	CTGGAGAAGACA-GCCATCCAAAAT	3.2 kb
265	02337-L02469	PAH exon 12	1743-1744	GATTGAGGTCTT-GGACAATACCCA	1.3 kb
292	02339-L01829	PAH exon 13	1829-1830	AGAAAATAAAGT-AAAGCCATGGAC	363.4 kb
		PAH stop codon	*1829-1831 (exon 13)*		
310 ¬	02340-L01834	IGF1 gene		AGGTAGAAGAGA-TGCGAGGAGGAC	

(a) The exon numbering used in this P055-D1 PAH product description and in the P055-D1 PAH Coffalyser.Net analysis sheet is identical to the exon numbering in the NCBI NG_008690.1 reference sequence. The exon numbering and NM sequence used is from 06/2018, but can be changed (e.g. by NCBI) after the release of the product description.

(b) Ligation sites of the P055 PAH MLPA probes are indicated according to Refseq sequence NM_000277.1, not the location from the start codon. The NM_000277.1 sequence contains 13 exons and is derived from the widely used U49897.1 sequence. These two sequences have the same length and the same location of the coding sequence.

(c) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of flanking and reference probes are unlikely to be related to the condition tested.

Related SALSA MLPA probemixes

P076 ACADVL-SLC22A5: Similar to PAH, defects in ACADVL are often detected by newborn screening.

References

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P055 Product history	
Version	*Modification*
D1	One PAH exon 6 probe added, one PAH exon 12 probe (wild-type sequence at R408W mutation) removed. One reference probe replaced.
C1	Seven PAH probes and eight reference probes replaced. New 88 and 96 nt control fragments (QDX2).
B1	An increase of PAH probes, from 13 to 22. New control fragments.
A1	First release.

Implemented changes in the product description
Version D1-03 – 17 July 2018 (04) - Product description restructured and adapted to a new template. - IVD use now includes Morocco. - Information about new and changed probes in version D1 compared to C1 removed from table 1 and 2. *Version D1-02 – 04 April 2017 (03)* - Product description adapted to a new template. - New publications added which used P055. *Version D1-01 - 18 January 2016 (02)* - Product description restructured and adapted to a new template. - Selected publications list updated. *Version 09 (2)* - Product description restructured. - Minor textual changes. *Version 08 (1)* - Product description completely rewritten and restructured. - Product description adapted to a new product version (version number changed, lot number added, changes in Table 1 and Table 2, new picture included). *Version 07 (48)* - Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added. *Version 06 (48)* - Information added about probes targeting HSS2 and HSS3 region. - Various minor textual changes. *Version 05 (48)* - Remark on RefSeqGene standard added below Table 2. - Various minor textual changes. - Ligation sites of the probes targeting the PAH gene updated according to new version of the NM_reference sequence.

More information: www.mlpa.com; www.mlpa.eu	
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IVD	EUROPE* CE 0344 MOROCCO
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and
members of the European Free Trade Association (EFTA).
The product is for RUO is all other European countries.